EXHIBIT B25

The role of talc powder exposure in ovarian cancer: mechanistic approach

Our laboratory's primary research has focused on investigating the role of oxidative stress in the pathogenesis of epithelial ovarian cancer (EOC) for many years. We have reported that EOC tissues and cells manifest a pro-oxidant state characterized by an increased expression of key pro-oxidant enzymes such as inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase, as well as an increase in nitric oxide (NO) levels [1-3]. Additionally, we have shown that EOC cells manifest lower apoptosis, which was markedly induced by inhibiting iNOS, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [2]. In an attempt to identify the mechanism of apoptosis in EOC cells, we examined the process of S-nitrosylation of caspase-3, which is known to inhibit its activity resulting in lower apoptosis. There was a significant increase in S-nitrosylation of caspase-3, which correlated with a significant decrease in caspase-3 activity in EOC cells. Myeloperoxidase (MPO) is a key oxidant enzyme that utilizes NO produced by iNOS, as a one-electron substrate generating $NO^{\bullet}$, a labile nitrosating species [4-7]. Indeed, we were the first to report that MPO was expressed by EOC cells and tissues [1]. Collectively, this work suggests that MPO is a key player in regulating apoptosis in EOC cells, but also highlights a possible cross-talk between iNOS and MPO [1]. Additional findings from our laboratory highlighted the potential benefits of the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer [8].

Epidemiologic studies have established the role of family history as an important risk factor for both breast and ovarian cancers [9]. A woman's risk for ovarian cancer is three times higher if she has a close relative who has had ovarian cancer. Genomic studies of high-grade serous cancer have identified molecular subtypes that are associated with distinct biology and clinical outcome. It is well known that germline mutations in BRCA1 or BRCA2 result in a predisposition to ovarian cancer at a rate of only 20-40%, suggesting the presence of other unidentified mutations in other predisposition genes [10-13]. Additional genetic variations, many of which have been identified in recent genome-wide association studies (GWAS), have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [12, 14, 15]. Thus, there is an emerging consensus that most of the genetic component of ovarian cancer risk is due to genetic polymorphisms that confer low to moderate risk [16]. Single nucleotide polymorphisms (SNPs) occur because of point mutations that are selectively maintained in populations that are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs [17]. Non-synonymous SNPs substitute encoded amino acids in proteins, and are more likely to alter the structure, function, and interaction of the protein [12, 18]. Therefore, SNPs are good candidates as disease-modifiers and have been associated with an altered cancer risk. Because reactive oxygen species (ROS) can cause severe damage to DNA, protein, and lipids they can be considered as an important class of carcinogens [19]. Therefore, antioxidant defense enzymes are of great importance to control the cellular redox level and regulate accumulation of ROS. Our recently published study found that chemoresistant EOC cells manifested specific point mutations, which are associated with altered enzymatic activity, in key redox enzymes that are not detected in sensitive counterparts [20]. Causality was established by the induction of point mutations that corresponded to known change of function SNPs in sensitive EOC cells, which resulted in a significant increase in the level of chemoresistance. These findings indicate that chemotherapy induces specific point mutations, which correspond to change of function SNPs, in key redox enzymes that contribute to the acquisition of chemoresistance in EOC cells, highlighting a potential novel mechanism. *Here, our objective is to determine whether talc can induce such mutations in the key redox enzymes, contributing to the oncogenic phenotype.*

The persistent generation of cellular reactive oxygen species (ROS) is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [21-24]. Talc and asbestos are both silicate minerals, however the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature [25]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response [25]. Inflammation has long been associated with cancer. Although there is strong epidemiological evidence to suggest an association between talc use and ovarian cancer, the direct link and precise mechanisms have yet to be elucidated.

Aim I: Determine the effect of talc on the redox balance in normal ovarian surface epithelial and ovarian cancer cells. To accomplish this aim, we will measure the activity and expression of select key oxidants and antioxidants in cell culture lysate from primary cultures of ovarian surface epithelial cells (n=3) as well as in ovarian cancer cell lines (n=3), as described in general methods, before and after exposure to talc. Cells will be exposed to increasing doses of talc (100, 200, 500 µg/mL) for 24, 48, and 72 hours. Based on our extensive published results, the following markers will be evaluated for activity and expression: NAD(P)H oxidase (NOX2 and NOX4), nitrate/nitrite, glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST), total glutathione (GSH), iNOS, MPO, catalase (CAT), superoxide dismutase (SOD), and 8-OHdG. Total RNA and protein will be extracted for the evaluation of marker levels will be assessed by a combination of real-time RT-PCR and ELISA. ***We expect that exposure of normal ovarian surface epithelial cells to talc will alter the redox balance to mimic that observed in ovarian cancer cells. Furthermore, we expect that talc will further alter the redox balance in ovarian cancer cells which will contribute to the maintenance and severity of the oncogenic phenotype as well as promote metastasis.*** We hope to accomplish this Aim by October 10th, in order to submit our findings to our premier society, *Society of Reproductive Investigation* (SRI).

This Aim is divided into 2 phases.
Phase I, PCR.
Estimated time to execute this Aim is 4 weeks.
Supplies $25,840
Labor $11,500
Data Analysis/Statistics $1500

Phase II, ELISAs.
ELISA kits are very expensive and we propose to run ELISAs only for makers that showed differential expression between treated and untreated.
Each ELISA will cost $10,500.

Aim II: Determine whether exposure to talc can induce point mutations that correspond to known SNPs in key oxidant and antioxidant enzymes as well as BRCA1/2, in *normal ovarian surface epithelial and* ovarian cancer cells. To accomplish this aim, we will perform SNP genotyping analysis before and after talc exposure (500 µg/ml, 72 hours) utilizing DNA isolated from epithelial ovarian cancer cell lines (n=8) and normal ovarian surface epithelial cell lines (n=3), as described in general methods. The TaqMan® SNP Genotyping Assay Set (Applied Biosystems, Carlsbad, CA) will

be used to genotype the SNPs, as previously described [20]. The Applied Genomics Technology Center (AGTC, Wayne State University, Detroit, MI) will perform this assay. Analysis will be done utilizing the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems). The SNPs will be selected based on the results of Aim I. We have previous analyzed the following SNPs in EOC cells and patient DNA: rs4673 (*CYBA*), rs4880 (*MnSOD),* rs2297518 (*NOS2*), rs3448 (*GPX1*), rs1001179 (*CAT*), rs2333227 (*MPO*), and rs1002149 (*GSR*) [20, 26]. Due to the known strong association between BRCA1/2 and ovarian cancer, we propose to analyze the following SNPs: dbSNP rs67284603, rs80357569, rs80359874 (BCRA1) and rs80359671, rs80359368, rs80359352 (BRCA2). ***We expect that exposure of normal ovarian surface epithelial cells to talc will induce specific point mutations in key redox enzymes as well as BRCA1/2, thereby acquiring a phenotype/genotype similar to ovarian cancer cells. Furthermore, exposure of ovarian cancer cells to talc will induce these mutations which may contribute to the maintenance and severity of the oncogenic phenotype as well as promote metastasis. Collectively, the results from Aim I and II will elucidate a potential mechanism by which talc powder exerts its oncogenic effects.***

Estimate time to execute this Aim is 3 weeks.
Phase I will be to treat and collect cells for analysis.
Supplies: $17,080
Labor $$8,625

Phase II: SNP analysis by Core Facility
This will cost $11,700

Aim III: Exposure to talc results in neoplastic transformation of normal ovarian surface epithelial cells. To accomplish this aim, we will assess the ability of talc exposure to cause neoplastic changes in normal ovarian surface epithelial cells (n=3) utilizing a neoplastic transformation assay, as previously described [27]. Moreover, we have recently established a mechanism of decreased apoptosis specific to epithelial ovarian cancer cells through S-nitrosylation of caspase-3 in response to a cross-talk between MPO and iNOS, key redox and inflammatory enzymes. In this aim we will determine whether this mechanism, a characteristic of ovarian cancer cells, will hold true for the transformed normal ovarian surface epithelial cells. Normal ovarian surface epithelial cell lines will be treated with talc (optimum dose and time point determined in Aim I), cells will be collected, washed and suspended in agar at 500 cells/well and layered on top of a base of 0.8% agar in a 96 well plate, per the manufacturer protocol (Cell Transformation Detection Assay, Millipore). The plates will be incubated at 37°C in a humidified incubator for 14-21 days. Colonies will be quantified a cell quantification solution and color change detected at 490nm. Both positive (MNNG and TPA) and negative controls (agar without cells) will be included. The activity and the expression of the following markers will be evaluated before and after exposure to talc; MPO, iNOS, caspase-3, apoptosis, and S-nitrosylation of caspase-3 utilizing a combination of ELISA, real-time RT-PCR and Western blot, all routinely established methods in our laboratory. ***We expect that exposure of normal ovarian surface epithelial cells to talc will result in neoplastic transformation of these cells over time, which is critical in establishing a cause and effect relationship. We also expect that exposure of normal ovarian surface epithelial cells to talc will result in increased caspase-3 S-nitrosylation and decreased apoptosis similar to what is observed in ovarian cancer cells.***

Estimate time to execute this Aim is 4 weeks.

Phase I will be the transformation assay
Supplies $12,400
Labor $7,500

Phase II will be the S-nitrosylation of caspase-3 assay/apoptosis
Supplies $6,500
Labor $6,300
Data Analysis/Statistics $900

General Methods: *All experiments will be performed in triplicate. Samples will be subjected to the following assays according to the manufacturer's protocols:*

Human EOC cell lines, MDAH-2774 (CRL-10303), SKOV-3 (HTB-77), OV90 (CRL-11732), TOV-21G (CRL-11730), TOV-112D (CRL-11731), OVCAR-3 (HTB-161), are obtained from American Type Culture Collection (ATCC), and are all cultured with media supplemented with fetal bovine serum (FBS, Innovative Research) and penicillin/streptomycin according to the manufacturers' protocols. The OV433 EOC cell line was a kind gift from Gen Sheng Wu at Wayne State University, Detroit, MI [28]. A2780 EOC cells are obtained from Sigma Aldrich and are cultured in HyClone RPMI-1640 (Fisher Scientific) with FBS, per the manufacturer's protocol.

Primary ovarian surface epithelial cells (n=3): Human primary ovarian surface epithelium (HOSEpiC, ScienCell Research Laboratories) were cultured with Ovarian Epithelial Cell Medium, as previously described [29]. Two additional ovarian surface epithelial cell lines will be obtained from Cell Biologics and ABM, and will be cultured per the manufacturer's protocols.

Protein Extraction: Total protein concentration of cell lysates will be measured with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) per the manufacturer's protocol. Cell lysates will be prepared as previously described [30].

Extraction of RNA and real-time RT-PCR. RNA will be extracted from EOC cells and their chemoresistant counterparts and will be utilized to determine the mRNA levels of β-actin, MPO, GPX, GSR, SOD, GST, NOX2, NOX4, CAT, and iNOS utilizing specific primers designed with the help of software program, Beacon Designer (Premier Biosoft) as we have previously described [1, 20, 30, 32, 33].

Detection of S-nitrosylation of caspase-3: S-nitrosylation will be determined with the S-nitrosylation Detection Kit (Cayman Chemical), per the manufacturer protocol in cell lysates from normal ovarian surface epithelial cells or ovarian cancer cells from the different treatments. Caspase-3 protein will be immunoprecipitated with anti-caspase-3 monoclonal antibody conjugated with protein A/G plus agarose beads. Biotinylated proteins will be separated by SDS-PAGE and detected using nitrosylation detection reagent I (HRP) according to the manufacturer's protocol as previously described [1].

ELISAs – Unless otherwise stated, all assays will be performed utilizing cell lysate according to manufacturer's protocols.

Apoptosis will be assessed by the TUNEL assay (Promega) and the Caspase-3 Colorimetric Activity Assay (Cayman Chemical) as we have previously described [3, 33]. Oxidant enzyme activity and levels will be determined with the Myeloperoxidase Enzyme Immunometric Assay Kit (Enzo Life Sciences) and the nitrate/nitrite colorimetric assay (Cayman Chemical) to measure the levels of stable NO by-products, NO_2^- and NO_3^-, as an indication of NO production, both as previously described [1, 8]. Antioxidant enzyme activities and levels will be determined using Catalase Assay Kit, Superoxide Dismutase Assay Kit, Glutathione assay kit, all

from Cayman Chemical, and the Glutathione Reductase Assay Kit, Glutathione Peroxidase Assay Kit and Glutathione S-Transferase Assay Kit, will be used per the manufacturer's protocol or as previously described [30, 34]. The DNA/RNA oxidative damage assay kit (Cayman Chemical), measures DNA/RNA oxidative damage in all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. This assay measures the amount of DNA/RNA tracer bound to oxidatively damaged guanine that forms a complex that is fixed to the microplate via a monoclonal antibody, which can be detected at 412 nm following an enzymatic reaction with Ellman's Reagent.

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